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14. ABSTRACT
Despite significant advances in the treatment, prostate cancer (PCa) remains a leading cause of cancer death among men. Androgen deprivation therapy (ADT) constitutes the main therapeutic option for patients with advanced PC. However, the major cause of death in men with metastatic prostate cancer involves progression to castration-resistant prostate cancer (CRPC). Characterizing mechanisms of resistance to ADT could enable the development of more effective therapeutic strategies. We performed a systematic genome-wide suppressor RNAi screen in the androgen-sensitive LNCaP cells, and identified genes whose silencing drives resistance to ADT in androgen-sensitive LNCaP cells, using nextgen sequencing. shRNA-mediated-knock-down and CRISPR-mediated knock-out *in vitro* experiments, as well as *in vivo* PCa xenografts, confirmed a role for the top hit INPP5A as a gene whose silencing modulates resistance to ADT in prostate cancer, through a mechanisms only partially AR-dependent.INPP5A gene was found deleted in CRPC patients, as well as INPP5A mRNA levels reduced in CRPC patient-derived xenografts, supporting the idea that INPP5A may prove clinical relevance for patients with castration-resistant PCa.

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1. INTRODUCTION

Prostate cancer is the second most common cause of cancer death among men in the United States. Prostate cancer malignant cells require androgen receptor (AR) signaling and the presence of androgens for their growth and survival [1]. Therefore, androgen deprivation therapy (ADT, castration) has been implemented as a first-line therapy for patients with metastatic disease [2]. Unfortunately, however, although almost all men with advanced prostate cancer initially respond to castration, the major cause of death in men with metastatic prostate cancer involves progression to castration-resistant prostate cancer (CRPC) [3].

Mechanisms underlying resistance to castration-based therapies in PCa have been intensely studied over the past years [3]. It is well established that the AR pathway plays a central role in the progression of PCa [4]. Indeed, CRPC cells maintain active AR signaling, despite castrated androgen levels [5, 6].

Recent therapeutic advances and clinical benefits for CRPC have been achieved by treatment with abiraterone, which inhibits the enzyme (CYP17) that catalyzes the formation of testosterone precursors [7, 8], or with antiandrogens, such as the second-generation AR antagonists MDV3100, approved for the treatment of CRPC in august 2012 [9, 10]. Other antiandrogens are in late-stage development, such as ARN-509, an anti-androgen with similar *in vitro* activity to MDV3100 but with greater *in vivo* activity in CRPC xenograft models [10, 11]. However, resistance/relapse of castration-resistant prostate cancer inevitably occurs even after treatment with these agents, and CRPC remains a formidable medical challenge.

RNA interference (RNAi) screens have recently emerged as a successfully new tool for a systematic study of resistance to anticancer agents [12, 13]. In this study, we performed an RNAi screen on the androgen-sensitive LNCaP cells, to identify genes whose silencing drives resistance to ADT. The characterization of novel mechanisms of resistance to ADT emerging from this screen, together with implementation of existing clinical data, could enable the development of more effective therapeutic strategies.

2. KEYWORDS

PCa – Prostate Cancer
CRPC – Castration Resistant Prostate Cancer
ADT – Androgen Deprivation Therapy
AR – Androgen Receptor
RPMI – Roswell Park Memorial Institute (culture **medium**)
CSS – Charcoal-Stripped Serum
RNAi – RNA interfering
shRNA – short hairpin RNA
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
FBS – Fetal Bovine Serum
GFP – Green Fluorescent Protein
LNCaP – Lymph Node Carcinoma of the Prostate
MDV3100 – Enzalutamide
mRNA – Messenger RNA
PSA – Prostate-Specific Antigen
qRT-PCR – Quantitative Real-Time PCR
CaMKII – Ca^{2+}/calmodulin-dependent protein kinase II

3. KEY RESEARCH ACCOMPLISHMENTS

As stated in the approved SOW, our goals were:

SPECIFIC AIM 1. To perform a systematic RNAi screen to identify loss of function mechanisms of resistance to androgen deprivation therapy.

Major task 1: Perform a systematic RNAi suppressor screen. As described in the annual report 2014, we performed a pooled genome-scale RNAi suppressor screen using the androgen-dependent LNCaP cell line cultured in charcoal-stripped serum (CSS), and identified a rank of genes whose silencing confers resistance to androgen deprivation *in vitro*. (Major task 1 successfully completed).

Major Task 2: Validation of candidate genes *in vitro*. As described in the previous report, LNCaP cells were used as cellular system to conduct validation experiments. Crystal violet assays performed over 3 weeks showed that most of the top hits emerging from the screen were successfully validated. Specifically, we described how we focused our validation and follow-up studies on the top hit membrane-associated type I inositol-1,4,5-trisphosphate (InsP3) 5-phosphatase (INPP5A). (Major task 2 successfully completed).

2a. Validate candidate genes on additional androgen-sensitive PCa cell lines. We wanted to expand the validation of top candidate hits using a second androgen-sensitive cell line. Culturing of the androgen sensitive cell line VCaP cells in androgen-deprived RPMI medium is highly challenging, thus, we obtained an additional cell line, LAPC-4, which contains a wild type AR and is an androgen dependent cell line, from our colleague Philip W. Kantoff of the Dana-Farber Cancer Institute [14]. Currently we are still optimizing the conditions to culture these cells in absence of androgens, since they require a minimal androgen concentration to be able to survive, even in regular FBS-RPMI. We expect to be able to address this point in a very short period of time.

Major Task 3: Validation of candidate genes *in vivo*. In parallel to our *in vitro* mechanistic work showing a robust increase in proliferation effect in absence of androgens following INPP5A knock-down, we have performed *in vivo* animal studies to determine the ability of LNCaP cells with reduced INPP5A levels to form tumors xenografts in mice. For this, $2e^6$ LNCaP cells stably expressing 2 INPP5A-specific shRNAs (#0515 or #0516) or control shLuciferase-LNcaP cells were injected subcutaneously in both flanks of IcrTac:ICR-*Prkdc*^{scid} mice (10 intact mice/clone and 10 mice to be castrated/clone -- 60 mice total) [15]. Tumors were allowed to form and reach ~1cm diameter, at which point a subset of animals were castrated, while another was left intact. [Note: the castration procedure was added to the animal protocol of Dr. Garraway's (mentor) laboratory and approved by the IACUC]. Mice undergoing castration were anesthetized using an Induction chamber Isoflurane machine. An approximately 0.7 cm incision was made in the skin overlying the ventral abdomen, and another incision was made in the peritoneum immediately internal to the 1st incision. One testis was pulled out of the incision and separated from its blood supply, then cut away. After restoring the remaining spermatic cord to the abdomen, the procedure was repeated with the second testis. Both the peritoneal and the skin incisions were closed with suture. Animals were kept on a heating pad and monitored until awake. This experiment resulted extremely challenging given the mortality associated with castration surgery, as well as the variability of tumor onset with LNCaP cells.

In the end, we were able to examine the number of mice/tumors per arm indicated in **Figure 1A**. We found that, while the growth of shLuc- control LNCaP tumors was drastically reduced by castration (**Fig. 1B**), both

shINPP5A#0515 (**Fig. 1B-left**) and #0516 (**Figure 1B-right**)-expressing tumors were able to continue to grow under castration conditions. The knock-down of *INPP5A* in LNCaP cells infected with control or INPP5A-specific shRNAs was confirmed by immunoblot analysis at the time of injection (**Fig. 1C**). **Figure 1D** shows the overall growth of intact or castrated mice for all the conditions at day 21, the time point that was reached by most mice.

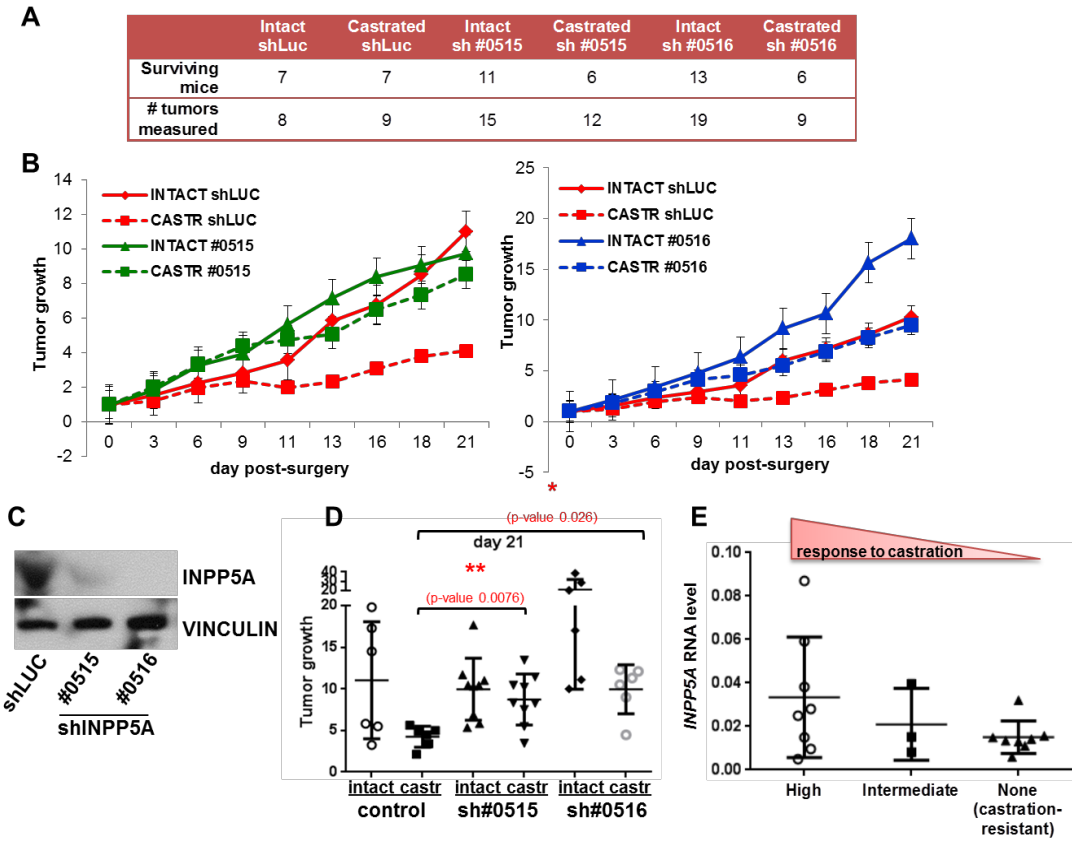

Figure 1. LNCaP-shINPP5A xenografts in castrated mice (A) Number of castrated/intact mice and number of tumors analyzed after subcutaneous injection of LNCaP cells expressing INPP5A-specific shRNAs (#0515 and #0516) or the control luciferase shRNA (shLuc). **(B)** Tumor-growth time course shown for LNCaP cells expressing INPP5A-shRNA #0515 (**left panel**) and #0516 (**right panel**). (**C**) Immunoblot analysis of INPP5A in LNCaP cells infected with control or INPP5A-specific shRNAs before the subcutaneous injection. **(D)** Summary of tumor growth for all conditions at day 21. **(E)** *INPP5A* mRNA levels are reduced in patient derived xenografts from CRPC patients who presented poor response to castration compared to those with better response.

In addition to PCa xenograft experiments, we also analyzed the expression of *INPP5A* mRNA in CRPC patient-derived xenografts. Here, we established a collaboration with Dr. Eva Corey at the University of Washington, whose lab obtained tumor samples from either radical prostatectomies or from rapid autopsy program and implanted them subcutaneously in 6 to 8 week old immune compromised male mice and allowed to grow. Upon collection of the established tumors, RNA was extracted and we determined the levels of *INPP5A* mRNA by real-time quantitative PCR. As shown in **Figure 1E,** we found that *INPP5A* levels were, in average, lower in tumors from patients who demonstrated intermediate- or no- response to castration, compared to tumors from highly-responsive patients.

Major Task 4: Analysis of gene expression of the validated resistance genes in human tumors.

As stated in the annual report 2014, we performed an analysis of copy number variation of the top 30 screen hits in metastatic CRPC, showing that and a number of them were found significantly deleted. Although we also have RNAseq data available relative to the same patient cohort, quality control analysis still needs to be completed for data analysis.

<u>SPECIFIC AIM 2</u>. To determine whether shRNAs identified by suppressor screen drive castration resistance through AR-dependent or -independent mechanisms.

Major task 1: Analysis of AR pathway activation in cells stably-expressing validated shRNAs

As described in the previous report, we assessed the level of AR pathway activation in LNCaP clones stably expressing shGFP or the INPP5A-specific shRNAs grown in CSS media by immunoblot analysis, and confirmed that silencing of *INPP5A* re-activates the AR pathway, despite the androgen of androgens (report 2014).

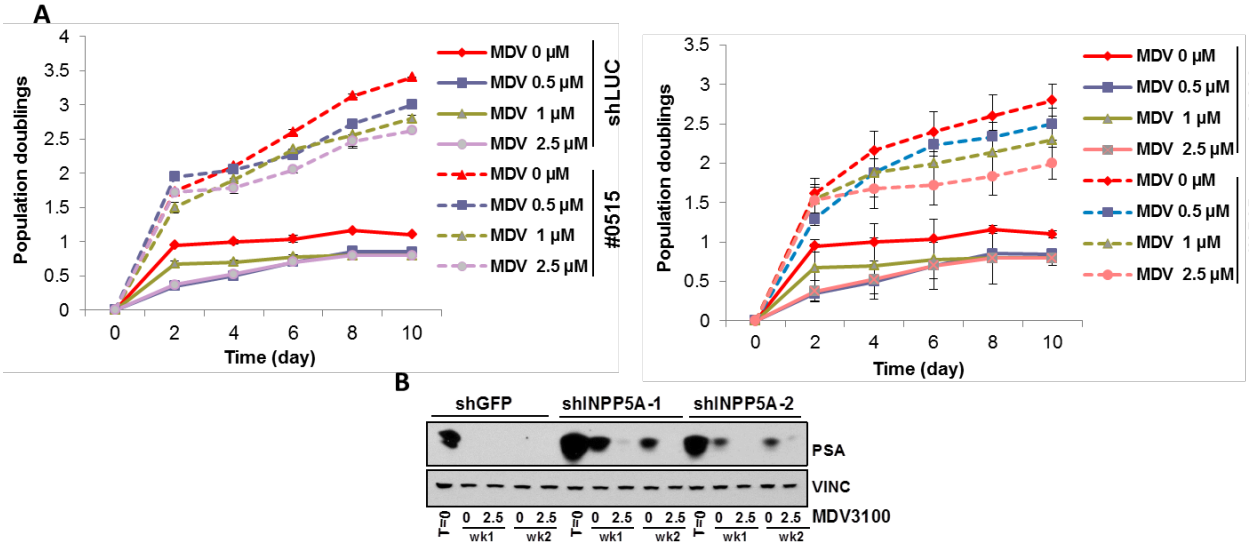

Figure 2. Loss of INPP5A confers resistance to the AR antagonist MDV3100 (A) INPP5A knock-down allows for LNCaP proliferation in the presence of AR inhibitor MDV3100. **(B)** PSA levels in LNCaP cells expressing *INPP5A* shRNas in the presence and absence of MDV3100.

To further characterize the dependencies on AR functions in the settings of INPP5A loss-driven resistance to ADT, we tested two AR antagonists, MDV3100 and ARN-509 [10]. To this aim, LNCaP cells infected with control or INPP5A-specific shRNAs were plated in 12-well plates in CSS in presence or absence of MDV3100 (2.5 µM), and counted every 2 days for 10 days. LNCaP cells expressing both shRNA #0515 (**Fig. 2A**, left panel--dash) and #0516 (**Fig. 2A**, right panel--dash) showed robust cell proliferation compared to cells infected with control shLuciferase when cultured in CSS. The addition of MDV3100 only partially reduced this effect. Similar results were obtained when cells were treated with a second AR antagonist, ARN-509, and cell proliferation was analyzed by crystal violet assay after 3 weeks in CSS (**Fig. 3A**).

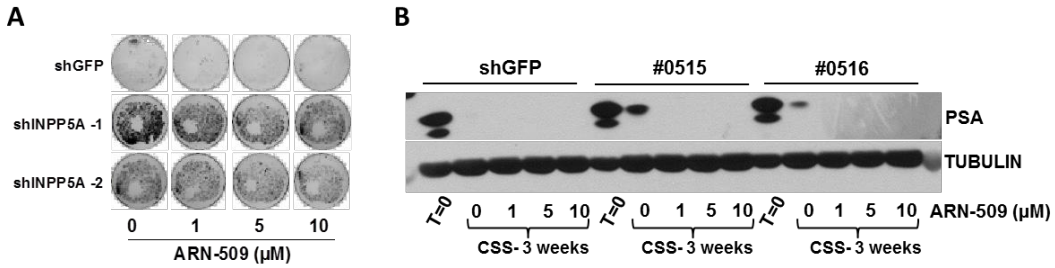

Figure 3. Loss of INPP5A confers resistance to the AR antagonist ARN-509 (A) Colony formation assays with LNCaP cells expressing INPP5A shRNAs in the presence of AR inhibitor ARN509. **(B)** PSA levels in LNCaP cells expressing *INPP5A* shRNAs in the presence and absence of ARN-509.

The ability of both MDV3100 and ARN-509 to inhibit the AR pathway was assessed by analyzing the suppression of PSA levels by immunoblot analysis in CSS in presence of the drugs. The results showed that in CSS conditions, *INPP5A* knockdown conferred robust proliferation of LNCaP cells in the presence of two AR inhibitors, MDV3100 (enzalutamide) and ARN-509, despite the loss of PSA expression, suggesting that an AR-independent mechanism might be promoting resistance following INPP5A loss (**Fig. 2B and 3B**).

Major task 2: To investigate whether activation of the AR pathway is necessary for shRNA-driven resistance.

Following the results described above using AR antagonists, we next tested the effect of directly targeting the AR with IPTG-inducible shRNAs. We identified two AR-specific shRNAs that dramatically decreased AR protein levels (#0030 and #0062) (**Fig. 4**). To test the ability of *INPP5A* knock-down to promote cell proliferation in CSS conditions, cells infected with control or AR-specific shRNAs were cultured in presence or absence of IPTG (4mM) and then re-infected with control or INPP5A-specific shRNAs. Stable clones following the two infections were cultured in CSS. Unfortunately, upon stimulation with IPTG, even the control cells displayed some cell death, thus complicating the interpretation of results.

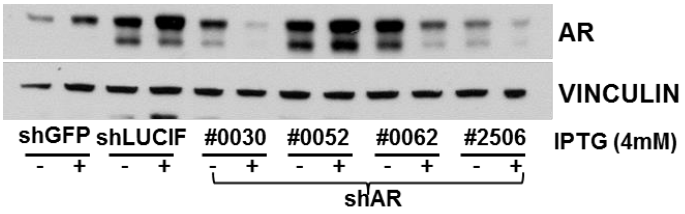

Figure 4. Knock-down of AR using IPTG-inducible constructs.

Next, we sought to achieve knock-out of *AR* by using targeted CRISPR genomic editing technology. Cas9-expressing LNcaP cells were infected with small guides targeting the *AR* gene. Unfortunately, only cells still expressing residual levels of AR were able to survive following selection (not shown). These results indicated that a complete knock-out of AR is not achievable, in accordance with the strong dependency of androgen-sensitive cells lines on AR activity.

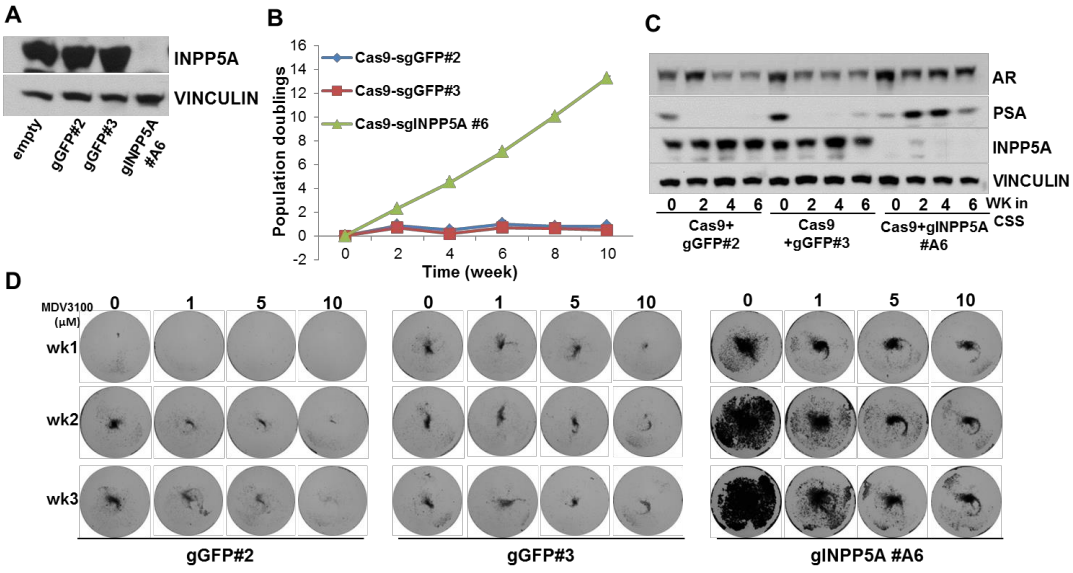

Figure 5. CRISPR-driven INPP5A knock-out (A) Immunoblot of INPP5A showing CRISPR-Cas9 knock out of endogenous *INPP5A* in LNCaP cells. **(B)** CRISPR-Cas9 KO of endogenous *INPP5A* in LNCaP cells drives proliferation in CSS medium. **(C)** AR and PSA levels in INPP5A-KO LNCaP cells cultured in absence of androgens for 6 weeks. **(D)** Crystal violet assay of Cas9-LNCaP INPP5A-KO grown in CSS in presence or not of enzalutamide at the indicated concentrations for 1-2-3 weeks.

2A. Generate LNCaP cells whose *INPP5A* genomic locus has been modified using targeted CRISPR genomic editing technology, thus resulting in CRISPR-driven *INPP5A* knock-out.

In addition to *in vitro* shRNA-based (previous report, and **Fig. 2A**) and *in vivo* (**Fig. 1**) validation assays, we undertook additional validation experiments employing the CRISPR- knock-out of the endogenous *INPP5A* gene. Five different small guides (sg) targeting the *INPP5A* gene were tested on LNCaP cells expressing Cas9 (pXPR_101 Cas9 lentiviral vector) (not shown). The guide conferring the most robust knock-out was selected for follow-up experiments (sgINPP5A #A6) (**Fig. 5A**). Population doublings counting of Cas9-LNCaP cells expressing two different sgGFP guides or the sgINPP5A A6 guide, confirmed the ability of *INPP5A* loss to promote robust cell proliferation in androgen-deprivation conditions (**Fig. 5B**). In addition, CRISPR-experiments demonstrated the ability of *INPP5A* silencing to maintain elevated expression of the AR transcriptional target PSA despite lack of external androgens (**Fig. 5C**). This confirmed our findings obtained with shRNA-experiments (previous report, and **Fig. 2B and 3B**). Proliferation assays in CSS in the presence of MDV3100 showed that the INPP5A-KO LNCaP cells are only partially sensitive to the AR antagonist (**Fig. 5D**), confirming the results obtained using INPP5A-specific shRNAs (**Fig. 2A**).

2B. Undertake detailed mechanistic studies to identify mechanism of resistance driven by INPP5A silencing.

As described in the previous report, INPP5A hydrolyzes Ins(1,4,5)P3 and Ins(1,3,4,5)P4, involved in release of ER and extracellular Ca^{2+}, which acts as a signaling second messenger (report 2014)[16, 17]. Loss of INPP5A, therefore, results in an increase of cytoplasmic Ca^{2+}. Therefore, we queried the role of Ca2+-mediated pathways in INPP5A-silencing-driven resistance to androgen deprivation. A Quantification of intracellular calcium was performed using the FURA-2 AM reagent, that is a cell-permeable acetoxymethyl (AM) ester binding to free Ca^{2+} [18]. Upon binding Ca^{2+}, the excitation spectrum of Fura-2 shifts to shorter wavelengths between 300 and 400 nm, while the peak emission remains steady

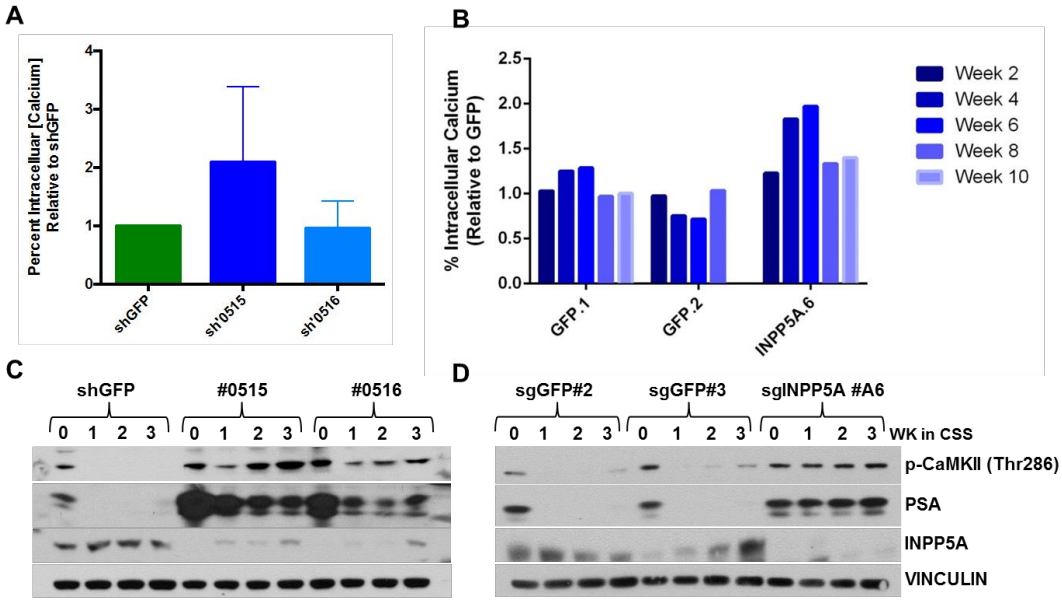

Figure 6. Analysis of Ca^{2+}-mediated pathways upon loss of INPP5A. Measurement of intracellular calcium in LNCaP cells expressing *INPP5A* shRNAs **(A)** or in Cas9-LNCaP cells expressing sgINPP5A **(B)** and cultured in CSS. **(C-D)** Immunoblot analysis of phospho-CamKII (Thr286) in INPP5A-KD LNCaP (C) or INPP5A-KO Cas9-LNCaP cells, cultured in CSS for 1-2-3 weeks.

around 510 nm, allowing accurate measurements of the intracellular Ca^{2+} concentration. Both shRNA- and CRISPR-experiments showed that silencing of INPP5A in CSS is paralleled by an increase in intracellular calcium compared to control cells (**Fig. 6A and 6B**).

The involvement of Ins(1,4,5)P3 and Ins(1,3,4,5)P4 in increased cytosolic Ca^{2+} influx led us to hypothesize that activation of the calcium/calmodulin–dependent protein kinase II (CaMKII) may play a central role in shINPP5A-driven resistance to androgen deprivation. Our hypothesis was based on reports showing that CaMKII overexpression increases secretion of PSA and sustains LNCaP cell growth in steroid-free condition by promoting escape from apoptosis [19, 20]. As greater amounts of calcium and calmodulin accumulate, CaMKII autophosphorylation occurs on the threonine 286. Once this residue has been phosphorylated, the inhibitory domain is blocked, allowing for permanent activation of the CaMKII enzyme. This enables CamKII to be active, even in the absence of calcium and calmodulin [21].

Interestingly, both INPP5A-KD LNCaP (**Fig. 6C**) and INPP5A-KO Cas9-LNCaP cells (**Fig. 6D**) displayed a significant increase in the phospho CamKII (Thr286) when cultured in the absence of androgens for several weeks, compared to control cells, only showing phosphorylated CamKII at time zero (FBS). These results indicated that CaMKII is permanently activated in LNCaP cells when INPP5A is silenced, suggesting a role for Ca^{2+}-mediated pathways.

In the past several years, progress has been made in understanding how Ca^{2+}/CaM regulates cell cycle transitions and affects the activation state of cyclin-dependent cdk complexes. Indeed, intracellular calcium levels are regulated as cells progress through the cell cycle, and Ca^{2+} is required early at G1 phase, as well as later near the G1/S boundary [22]. Experimental evidences strongly suggest that these Ca^{2+}/CaM-dependent pathways directly or indirectly regulate cyclin D1/cdk4 activity [23]. Therefore, we hypothesized that following loss of INPP5A, activation of cell cycle components might occur in response to an increase of intracellular Ca^{2+} and CaMKII activation, thus pushing cells through cell cycle transitions and division, even in absence of androgens.

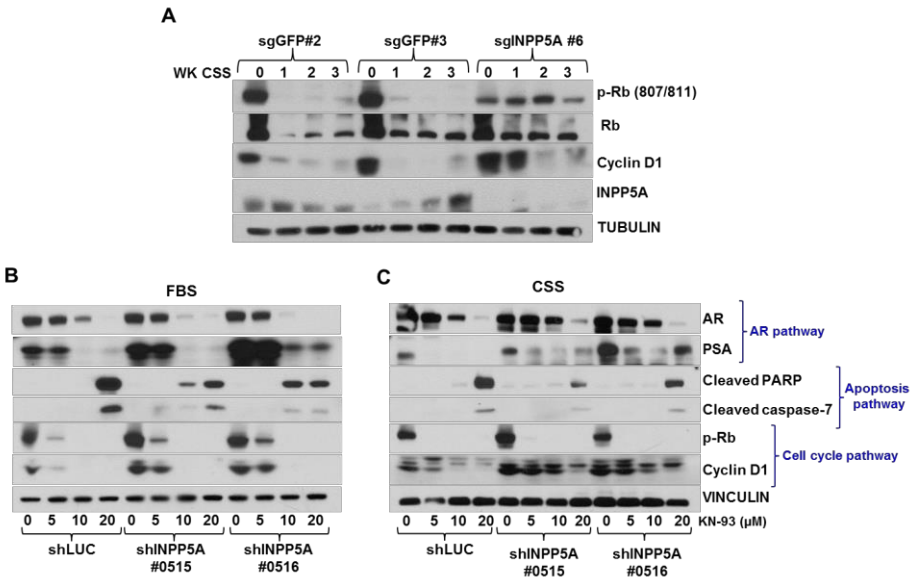

Figure 7. Correlation between Ca^{2+}/CaMKII pathway and cell cycle components **(A)** Western blot analysis of cyclin D1 and total and phospho-Rb in INPP5A-KO LNCaP cells cultured in CSS over 3 weeks. **(B-C)** Western blot analysis of AR pathway (AR, PSA), apoptosis pathway (cleaved PARP and caspase-7), and cell cycle pathway (p-Rb, cyclin D1) of INPP5A-KD LNCaP cells cultured in FBS (B) or CSS (C) and treated with KN-93 at the indicated concentrations for 48 hours.

To test this hypothesis, we first analyzed the levels of cyclin D1 and Rb activation in INPP5A-null LNCaP cells cultured in CSS for 1-2-3 weeks. **Figure 7A** shows that CRISPR-driven INPP5A-KO LNCaP cells do retain phosphorylated retinoblastoma on Serine 807/811 residues, as well as higher cyclin D1 levels,

compared to control cells, when cultured in CSS. Phosphorylation of Rb by cyclin D-cdk4/6 complex is required for its inactivation and subsequent cell cycle progression [24]. Thus, these results may indicate that, despite the absence of androgens, loss of INPP5A might promote cell cycle progression in PCa cells through cyclins/Rb modulation via Ca^{2+}/CaMKII pathway.

To further test this, INPP5A-shRNA expressing LNCaP cells were treated with increasing concentrations of the CaMKII inhibitor KN-93 and cultured in FBS or CSS in presence or absence of the drug (5-10-20 μM). In parallel, the KN-93 inactive analogue, KN-92, was used as control (data not shown). KN-93 is reported to suppress the expression of PSA, as well as to induce apoptosis in LNCaP cells [20]. Accordingly, control cells displayed suppressed PSA in response to the drug, as well as apoptosis markers, cleaved PARP and cleaved caspase-7, in either FBS (**Fig. 7B**) and CSS (**Fig. 7C**). Conversely, however, cells expressing INPP5A shRNAs showed reduction of PSA and induction of apoptosis at a much lower extent (**Fig. 7B** and **7C**), indicating that cells displaying increased CaMKII activation might be more resistant to the inhibitor. To test whether CaMKII might be the key mediator of cell cycle progression, we analyzed cyclin D1/Rb pathway in INPP5A-silenced LNCaP cells, in presence of KN-93. We found that cells expressing INPP5A shRNAs retain much more cyclin D1 in response to the drug, even more dramatically when cultured in CSS (**Fig. 7B** and **7C**). In addition, more phospho-Rb was detected in presence of the drug in FBS, compared to control cells.

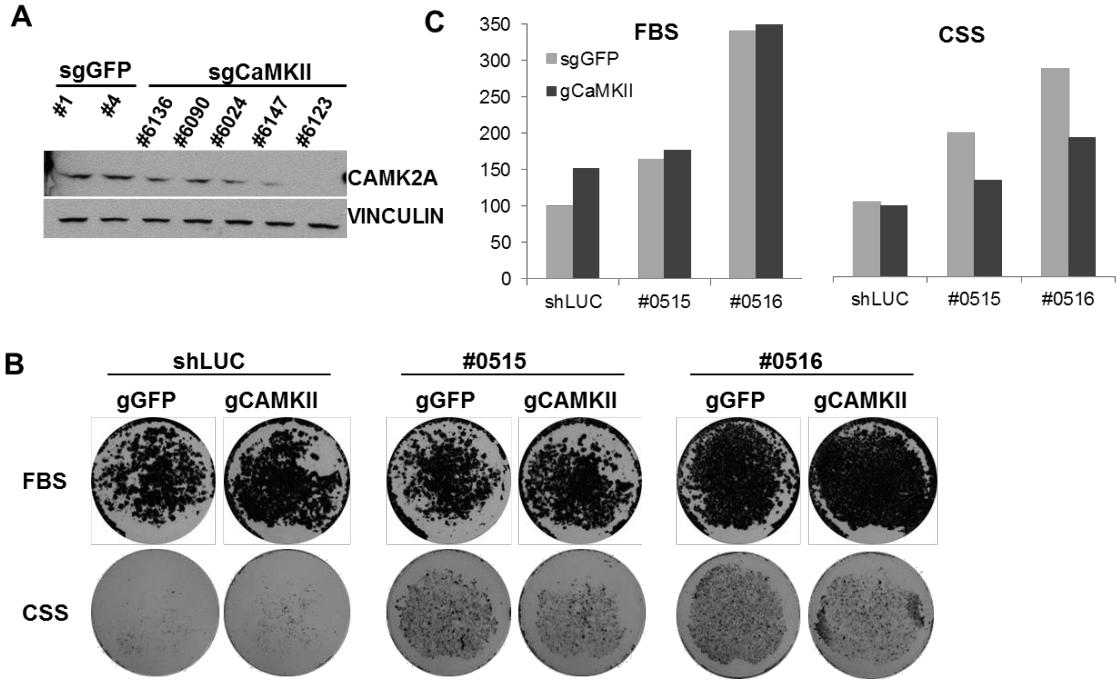

Figure 8. CRISPR-driven CaMKII knock-out (A) CaMKII protein levels in Cas9-LNCaP cells infected with control or CaMKII-specific guides. **(B)** Crystal violet assay of Cas9-LNCaP cells infected with control (GFP) or CaMKII-specific guide (#6123), and then re-infected with control (LUC) or INPP5A-specific shRNAs (#0515 and #0516), cultured 3 weeks in FBS or CSS. **(C)** Cell proliferation relative to figure B calculated relative to the control shGFP/shLUC by measuring absorbance of dissolved crystal violet at 595 nm.

Overall, these data indicate that LNCaP cells with loss of INPP5A display maintained active AR pathway, as well as activated Ca^{2+}/CaMKII pathway. In addition, these cells show activation of cells cycle components indicative of increased G1/S progression, despite the absence of androgens. These results are very interesting giving the fact that androgen deprivation therapy (ADT) induces growth arrest by inducing G1/S block, reduced cyclin-dependent kinase activity, and hypophosphorylated Rb [25]. In response to CaMKII inhibitor,

INPP5A-KD cells showed reduced suppression of AR pathway and cyclin D activation, as well as reduced induction of apoptosis compared to control cells, indicating that a hyperactive Ca^{2+}/CaMKII pathway might bypass the effect of drug.

To further characterize the role of CaMKII in INPP5A-driven resistance to ADT, we generated CaMKIIA-null LNCaP cells, by infecting Cas9-LNCaP cells with different CaMKIIA-specific RNA guides (**Fig. 8A**). The guide #6123 was chosen for follow-up experiments, given the robust knock-out achieved.

LNCaP cells displaying CaMKIIA knock-out were re-infected with control or INPP5A-specific shRNAs, and plated in FBS or CSS for 3 weeks. Proliferation was analyzed by crystal violet staining, showing that only a partial growth reduction in INPP5A-KD cells in CSS was observed when CaMKIIA was knocked-out, while no differences were observed in FBS (**Fig. 8B and 8C**). These results indicate that CaMKII might be only one of more mediators promoting INPP5A-resistance in this context, and that more Ca^{2+}-dependent or –independent mechanisms will need to be investigated.

We are currently working on generating LNCaP cells either CaMKIIA- and CaMKIIB-null, since both the isoforms have been reported to induce growth in absence of androgens [19], to determine whether the concomitant knock-out of the two isoforms will completely revert the INPP5A-mediated resistance phenotype.

MILESTONE ACHIEVED:

1. Validation of INPP5A loss as mediator of resistance to androgen-deprivation *in vitro* and *in vivo*.
2. Characterization of the response of INPP5A-silenced LNCaP cells to AR antagonists.
3. Generation of LNCaP cells whose *INPP5A* genomic locus has been modified using targeted CRISPR genomic editing technology, thus resulting in CRISPR-driven *INPP5A* knock-out.
4. Identification of candidate molecular pathways driving the INPP5A-mediated resistance in LNCaP cells displaying either *INPP5A* knock-down or knock-out.

CONCLUSIONS:

In conclusion, during the 2-year reporting period, we performed a genome-scale small-hairpin RNA (shRNA) screen on a prostate cancer cell model (LNCaP) that requires androgens for survival, and identified a rank of genes whose silencing (loss-of-function) drives proliferation in the absence of androgens. Importantly, some of the validated hits showed copy number deletion in CRPC patients. Among these, we identified the promyelocytic leukemia zinc finger (PLZF), also known as BTB-containing protein 16 (*ZBTB16*), as a tumor suppressor gene in metastatic CRPC and mediator of resistance to ADT (Chen-Lin Hsieh, **Ginevra Botta** et al., *Canc Res*, 2015;attached).

In addition, our work has identified the inositol phosphatase INPP5A as one of the strongest hits, and whose silencing sustains the growth of LNCaP cells and an active AR pathway in absence of androgens. Characterization of the response of cells lacking INPP5A to androgen receptor antagonists showed that loss of INPP5A can drive resistance through a mechanism only partially AR-dependent. Dissection of calcium signaling pathways implicated CaMKII as a candidate mediator of INPP5A-mediated resistance, as well as some cell cycle mediators involved in G1/S checkpoint transition.

The identification of novel mediators driving resistance to ADT in prostate cancer may open completely new insights, ultimately leading to new prospects of combination studies.

ACCOMPLISHMENTS TO ACHIEVE PRIOR TO PUBLICATION:

1. Addressing experiments of sufficiency and dependency for CaMKIIA and CaMKIIB in INPP5A-silenced cells.
2. Analyze the response of resistant cells to cdk4/6 inhibitors, in order to ultimately identify mediators of this pathway potentially targetable.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT:

As stated in the approved SOW, our goals were:

Major Task 1: Training and educational development in prostate cancer research

To execute the loss-of-function screening, I exploited the unique resources of DFCI and the Broad Institute, (Dr. Garraway is a Senior Associate Member of the Broad Institute), and I tightly collaborated with expert mentors of the Broad Institute genomic perturbation platform. To set up both the RNAi screening optimization and execution accordingly to the platform guidelines, I was constantly mentored by experts of the platform. In addition, the Broad institute gave us the opportunity to leverage constant collaborations with experts in cancer genomics, molecular oncology, prostate oncology, computational biology and statistics.

Part of my training in cancer biology has included the attendance of regular seminars and conferences. Indeed, each Tuesday I have attended the meeting of the Cancer Program of the Broad Institute, and on Tuesday afternoon the institute-wide series of Seminars in Oncology at DFCI, where invited speakers present their latest work. Moreover, at the Broad Institute I have attended a monthly "Resistance meeting", where postdoctoral fellows present their work focused on the study of resistance to anticancer treatment in different cancer types, and a monthly "Genomic Perturbation Platform meeting", where gain- or loss-of-function screening-based projects, executed on different cancer models, are presented.

Dr. Garraway and Dr. Hahn have consolidated several platforms at DFCI/Broad Institute to create an integrated approach for the study of resistance mechanisms to anticancer agents in specific cancers, including prostate cancer. In a monthly meeting under the direct supervision of both Dr. Garraway and Dr. Hahn, post-docs and graduate students from the two labs had the opportunity to discuss on the progress of projects exclusively focusing on prostate cancer.

Finally, in Dr. Garraway's laboratory, we have a weekly meeting on Wednesday morning dedicate to critically discuss each ongoing project in the lab. In addition to these meetings, I have met formally with Dr. Garraway at least twice per month, to discuss new results and plans in detail, and to receive both scientific and technical advices. Importantly, I also had the opportunity to review and criticize papers from high impact journals, focusing on prostate cancer research, under the direct Dr. Garraway's supervision.

The attendance to all the mentioned meetings has guaranteed a continued collaborations and mentorship, significantly improving my development in the area of prostate cancer molecular oncology.

Besides the attendance of meetings, I have attended the international conference "American Association for Cancer Research" (AACR) annual meeting (april 5-9, 2014, San Diego), the "Ninth Annual Broad Institute Scientific Retreat" and the Tenth Annual Retreat (November 12-13, 2013/ November 17–18, 2014

Boston), and the "STARR Cancer Consortium Retreat" (September 23-24, 2013, Cold Spring Harbor Laboratory).

DISSEMINATION OF RESULTS TO COMMUNITIES OF INTEREST:

To achieve the stated goals, I have presented my work at several seminars at DFCI/Broad institute department meetings:

- Cancer Program of the Broad Institute
- Genomic Perturbation Platform monthly meeting (twice)
- Garraway lab weekly meeting (every three months)

In addition, I have presented a poster at the following meetings:

- "American Association for Cancer Research" (AACR) annual meeting
- "STARR Cancer Consortium Retreat"

During the STARR Cancer Consortium retreat, I had the opportunity to disseminate our results to five biomedical research institutions — Memorial S loan Kettering Cancer Center, the Broad Institute of MIT and Harvard, Cold Spring Harbor Laboratory, The Rockefeller University, and Weill Cornell Medical College. During the AACR annual meeting, I could interact and exchange among scientists from all over the world.

In addition, I've been selected for oral presentation at the Eleventh Annual Broad Retreat, that will be held December 14–15 2015.

MILESTONE ACHIEVED:

1. Presented research at the monthly department group meetings
2. Attended conferences and retreats
3. Presentation of project data at a national meeting

4. IMPACT

IMPACT ON THE DEVELOPMENT OF THE PRINCIPAL DISCIPLINE(S) OF THE PROJECT:

The described project developed during the 2-year reporting period has leveraged some technical skills critical in the field of resistance of prostate cancer to castration-based therapy. The main skills during the first year included included lentiviral shRNA-based systematic functional studies, sequencing methods, and analysis of patient tissue collection. During the second year of the reporting period, the completion of this project has required additional crucial skills, such as analysis of patient-derived xenografts (PDX) and castration surgery on mice. Overall, the funding of this project has allowed an integration of novel loss-of-function *in vitro* data with clinical recurrent alterations in CRPC. Therefore, the work conducted in the past two years may lead to significant advances in the knowledge of dysregulation of mechanisms driving resistance, ultimately opening new perspective for the application of durable therapeutic approaches for PCa.

IMPACT ON OTHER DISCIPLINES: Nothing to Report

IMPACT ON TECHNOLOGY TRANSFER: Nothing to Report

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY: Nothing to Report

5. CHANGES/PROBLEMS

Nothing to report

6. PRODUCTS

PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS:

Chen-Lin Hsieh, **Ginevra Botta**, Shuai Gao, Tiantian Li, Eliezer M.Van Allen, Daniel J. Treacy, Changmeng Cai, Housheng Hansen He, Christopher J. Sweeney, Myles Brown, Steven P. Balk, Peter S. Nelson, Levi A. Garraway, and PhilipW. Kantoff; **PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration-Resistant Prostate Cancer, Is a Mediator of Resistance to Androgen Deprivation Therapy**; *Cancer Research*; 75(10) May 15, 2015(1945-1948) (Published)

JOURNAL PUBLICATIONS: Nothing to report

BOOKS OR OTHER NON-PERIODICAL, ONE-TIME PUBLICATIONS: Nothing to report

OTHER PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS:

Oral and poster presentation made in the last year are listed above (section "dissemination of results to communities of interest"). One publication is attached to this document.

WEBSITE OR OTHER INTERNET SITE(S): Nothing to report

TECHNOLOGIES OR TECHNIQUES: Nothing to report

7. INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES: Nothing to report

OTHER PRODUCTS:

Research material: generation of LNCaP cells stably expressing screen top hits shRNAs.
Generation of LNCaP cells stably expressing Cas9 gene and INPP5A small RNA guides

7.PARTECIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

INDIVIDUALS THAT HAVE WORKED ON THE PROJECT: Ginevra Botta; NO CHANGE

CHANGE IN THE ACTIVE OTHER SUPPORT OF THE PD/PI, OR SENIOR/KEY PERSONNEL SINCE THE LAST REPORTING PERIOD: Nothing to report

OTHER ORGANIZATIONS INVOLVED AS PARTNERS: Nothing to report

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: Nothing to report

QUAD CHARTS: Nothing to report

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APPENDICES

Chen-Lin Hsieh, **Ginevra Botta**, Shuai Gao, Tiantian Li, Eliezer M.Van Allen, Daniel J. Treacy, Changmeng Cai, Housheng Hansen He, Christopher J. Sweeney, Myles Brown, Steven P. Balk, Peter S. Nelson, Levi A. Garraway, and PhilipW. Kantoff; **PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration-Resistant Prostate Cancer, Is a Mediator of Resistance to Androgen Deprivation Therapy**; *Cancer Research*; 75(10) May 15, 2015(1945-1948)

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Priority Report

Cancer Research

PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration-Resistant Prostate Cancer, Is a Mediator of Resistance to Androgen Deprivation Therapy

Chen-Lin Hsieh[1], Ginevra Botta[1,3], Shuai Gao[4], Tiantian Li[1], Eliezer M. Van Allen[1,2], Daniel J. Treacy[1], Changmeng Cai[4], Housheng Hansen He[5,6], Christopher J. Sweeney[1], Myles Brown[1,2], Steven P. Balk[4], Peter S. Nelson[7], Levi A. Garraway[1,3], and Philip W. Kantoff[1]

Abstract

Whole-exome sequencing of metastatic castration-resistant prostate cancer (mCRPC) reveals that 5% to 7% of tumors harbor promyelocytic leukemia zinc finger (PLZF) protein homozygous deletions. PLZF is a canonical androgen-regulated putative tumor suppressor gene whose expression is inhibited by androgen deprivation therapy (ADT). Here, we demonstrate that knockdown of PLZF expression promotes a CRPC and enzalutamide-resistant phenotype in prostate cancer cells. Reintroduction of PLZF expression is sufficient to reverse androgen-independent growth mediated by PLZF depletion. PLZF loss enhances CRPC tumor growth in a xenograft model. Bioinformatic analysis of the PLZF cistrome shows that PLZF negatively regulates multiple pathways, including the MAPK pathway. Accordingly, our data support an oncogenic program activated by ADT. This acquired mechanism together with the finding of genetic loss in CRPC implicates PLZF inactivation as a mechanism promoting ADT resistance and the CRPC phenotype. *Cancer Res; 75(10); 1944–8. ©2015 AACR.*

Introduction

A long-standing challenge in the management of prostate cancer is the development of resistance to androgen deprivation therapy (ADT), a standard treatment to disrupt the androgen receptor (AR) signaling pathway, because AR has a profound effect on prostate carcinogenesis through the regulation of transcriptional networks, genomic stability, and gene fusions (1). Although ADT is initially effective and presumably extends the survival of most prostate cancer patients, prostate cancer inevitably becomes resistant to ADT and castration-resistant prostate cancer (CRPC) emerges (2). Newer agents targeting the androgen signaling axis (AR-targeted therapies), such as abiraterone and enzalutamide, have yielded improved outcomes for patients with CRPC. Unfortunately, not all patients with CRPC respond to these AR-targeted therapies, and moreover, these agents are not curative in this setting (3). The main subset of mechanisms of resistance to these antagonists involves the AR signaling pathway, including AR gene overexpression, gain-of-function mutations, constitutively active AR splice variants, dysregulation of its coregulators, and *de novo* androgen synthesis (4). Additional categories of resistance mechanisms consist of de-repression of progrowth pathways in response to ADT (5) or transformation to a distinct, androgen, and AR-indifferent cell state (4).

The recent surge of genomic and transcriptomic information may permit molecular classification of CRPC and future clinical development of precision medicine based on predictive biomarkers (5). Intriguingly, whole-exome sequencing of metastatic CRPC (mCRPC) revealed that 5% to 7% of tumors harbor promyelocytic leukemia zinc finger (PLZF) focal homozygous deletions. PLZF, also known as BTB-containing protein 16 (*ZBTB16*), was originally identified as a gene fused to RARα in acute promyelocytic leukemia patients (6). PLZF has been shown to play an important role in the regulation of major developmental and biologic processes and carcinogenesis as a tumor suppressor gene, since it regulates the cell cycle and apoptosis in various cell types (7). Overexpression of PLZF was shown to inhibit cellular proliferation in AR-positive LNCaP and AR-negative DU-145 prostate cancer cell lines (8, 9). Herein, our data show that PLZF emerged as the top gene from an AR cistrome analysis, credentialing PLZF as an androgen-regulated putative tumor suppressor gene in prostate cancer. Accordingly, we report a

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

C.-L. Hsieh and G. Botta contributed equally to this article.

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resistance mechanism to ADT mediated by PLZF, which appears to result from the activation of progrowth pathways in response to ADT. Furthermore, the findings of PLZF genetic loss in mCRPC tumors support that PLZF may be an important mediator in a subset of CRPC tumors.

Materials and Methods

Cell culture, lentiviral infection, and xenografts

LNCaP/22Rv1 and VCaP cells were cultured in RPMI1640 and DMEM medium with 10% FBS. 22Rv1 xenografts were established in the flanks of male nude mice by injecting approximately 2 million stable 22Rv1 cells with shCtrl or shPLZF knockdown in 50% Matrigel 3 days after castration. Tumors were measured 3 times every week and harvested after 3 weeks. All animal experiments were approved by the Beth Israel Deaconess Institutional Animal Care and Use Committee and were performed in accordance with institutional and national guidelines

Cell proliferation (crystal violet staining/WST1)

Cell growth was examined using the crystal violet (CV) staining and WST1 assays (Roche) following the manufacturer's protocol. CV was dissolved in 10% acetic acid and cell proliferation calculated relative to the negative control cells, by measuring the absorbance at 595 nm.

qRT-PCR, immunoblotting, and immunohistochemistry

RNAs were extracted using TRIzol according to the manufacturer's protocol. Primers are listed in Supplementary Text. qPCR data are represented as mean ± STD for more than 3 replicates. Blots were incubated with anti-PLZF (MAB2944; R&D Systems), anti-actin (A5316; Sigma), total p44/42 MAK (Erk1/2; 4695; Cell Signaling), or phospho-p44/42 MAK (Erk1/2; 4370; Cell Signaling). Paraffin sections underwent antigen retrieval and were subjected to the staining protocol using Dako EnVision+System-HRP 3,3′-diaminobenzidine (DAB). Anti-PLZF (MAB2944; R & D Systems), anti-Ki67 (Dako), or nonspecific IgG was then added overnight at 4°C. Sections compared in each figure were stained at the same time and photographed under identical conditions.

Chromatin immunoprecipitation assay and ChIP-Seq data analysis

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (10). The PLZF antibody (MAB2944; R&D Systems) or nonspecific IgG was used for ChIP. ChIP-Seq raw data were mapped by Bowtie 2 with default parameters. The identification of ChIP-seq peaks (bound regions and summit) was performed using MACS (PMID: 18798982). Regions of enrichment comparing to input control exceeding a given threshold ($P < 1e{-}5$) were called as peaks. The primers for qPCR are provided in the Supplementary Text.

Gene expression experiments and analysis

LNCaP cells were transfected with either control shRNA (shCtrl) or shRNAs targeting PLZF (shPLZF). Forty-eight hours after shRNA transfection, total RNA was isolated and hybridized to Affymetrix human U133 plus 2.0 expression array (Affymetrix). Raw data are preprocessed using RMA (PMID: 12538238) and the cutoff of 1.5-fold change, and P value of <0.05 is applied for differential expressed gene analysis.

Results and Discussion

The hope of precision medicine is to tailor treatment based on each patient's genomic and transcriptomic characteristics. This approach has proven to be challenging for the management of prostate cancer because of the paucity of actionable mutations found thus far. The recent finding (11) that 7% (4/61) of mCRPC tumors harbored PLZF homozygous deletions captured our interest (Fig. 1A). Indeed, homozygous deletion of PLZF was further seen in two independent cohorts: 6% (4/63) and 5% (8/152) from the University of Washington and the Stand Up to Cancer/

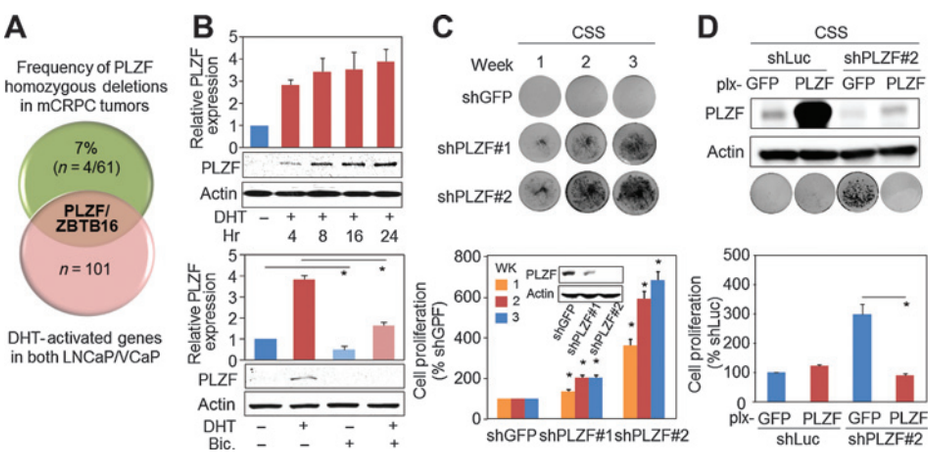

Figure 1.
PLZF is an androgen-regulated gene involved in growth suppression. A, Venn diagram showing the frequency (%) of PLZF homozygous deletions (n = homozygous deletions/total mCRPC tumors; ref. 11) and PLZF as a putative tumor suppressor gene with strongest AR binding merged from two AR cistrome datasets. B, qRT-PCR and Western blotting were used to measure PLZF mRNA and protein expression of LNCaP cells, which were cultured in CSS, followed by 10 nmol/L of DHT and/or 10 μmol/L of bicalutamide (Bic) treatment. The colonies were stained by CV and photographed. C and D, the efficiency and efficacy of PLZF shRNA knockdown (C) and ectopic re-expression of PLZF (D) were measured by Western blot. Each column was relative to the corresponding first column and shown as mean ± SD ($n \geq 3$). *, $P < 0.05$.

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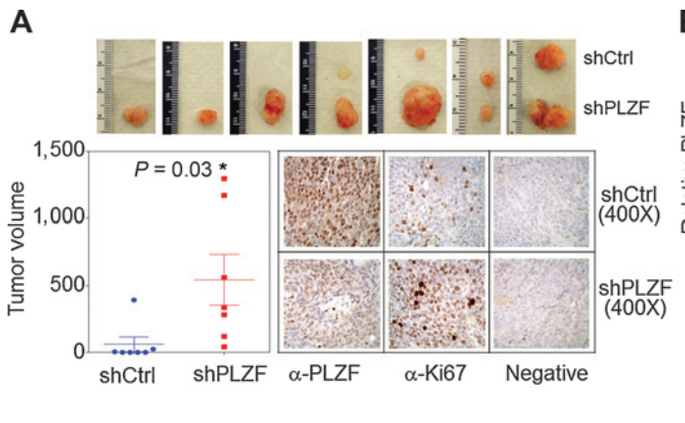

Figure 2.
PLZF functions as a tumor suppressor *in vivo*. A, tumor formation assays of castrated male nude mice injected with shCtrl and PLZF stable silencing 22Rv1 cells. Bottom right, averaged xenograft tumors (mean ± SEM); left, PLZF and Ki67 immunohistochemistry were used to monitor the efficacy of PLZF knockdown and cell proliferation in 22Rv1 xenografts. B, PLZF gene expression from 27 hormone-sensitive prostate cancers (HSPC) and 29 bone mCRPCs.

Prostate Cancer Foundation (SU2C/PCF), which will be part of a larger SU2C genomic landscape article (personal communications). This prompted us to explore the role of PLZF in prostate cancer. Here, we postulated that because PLZF was androgen regulated, AR might activate PLZF, an intermediate tumor suppressor gene that might derepress an oncogenic program with androgen depletion. This was based on the observation that ADT induces the expression of androgen-repressed genes that normally regulate androgen synthesis, DNA replication, and cell cycle progression in CRPC models (12).

Taking an agnostic approach, we sought androgen-regulated candidate tumor suppressor genes. We compiled two AR cistrome datasets and showed that PLZF was the canonical tumor suppressor gene with strongest androgen-induced AR recruitment to its putative enhancer regions in two androgen-dependent prostate cancer cell lines, LNCaP and VCaP (Supplementary Figs. S1 and S2A), implying that the tumor suppression function of PLZF may be diminished upon ADT treatment.

The androgen-stimulated effect on PLZF expression was demonstrated in LNCaP, VCaP, and 22Rv1 cells (Fig. 1B; Supplementary Fig. S2B and S2C). More importantly, PLZF expression was repressed by an antiandrogen (bicalutamide) in LNCaP cells (Fig. 1B, bottom).

To explore the tumor suppressive function of PLZF on prostate cancer, we examined the biologic effect of altered PLZF expression on cell growth in androgen-depleted condition. Knockdown of PLZF using four different shRNA constructs (shPLZF#1∼#4) promoted androgen-independent growth in LNCaP cells (Fig. 1C; Supplementary Fig. S3A). Conversely, re-expression of PLZF reversed the androgen-independent growth mediated by PLZF depletion (Fig. 1D).

To determine whether PLZF perturbation might promote androgen-independent growth *in vivo*, we also analyzed the growth of 22Rv1 prostate cancer xenograft expressing PLZF shRNAs, in castrated nude mice. Consistent with the *in vitro* observations, PLZF knockdown enhanced tumor formation in castrate levels of androgen (Fig. 2A). Altogether, our results show that PLZF is a putative AR-regulated tumor suppressor gene.

Next, we interrogated the extent to which PLZF expression might be altered in patient-derived CRPC tumor samples and found that mean PLZF expression was significantly lower in CRPC bone metastases compared with primary tumors (Fig. 2B),

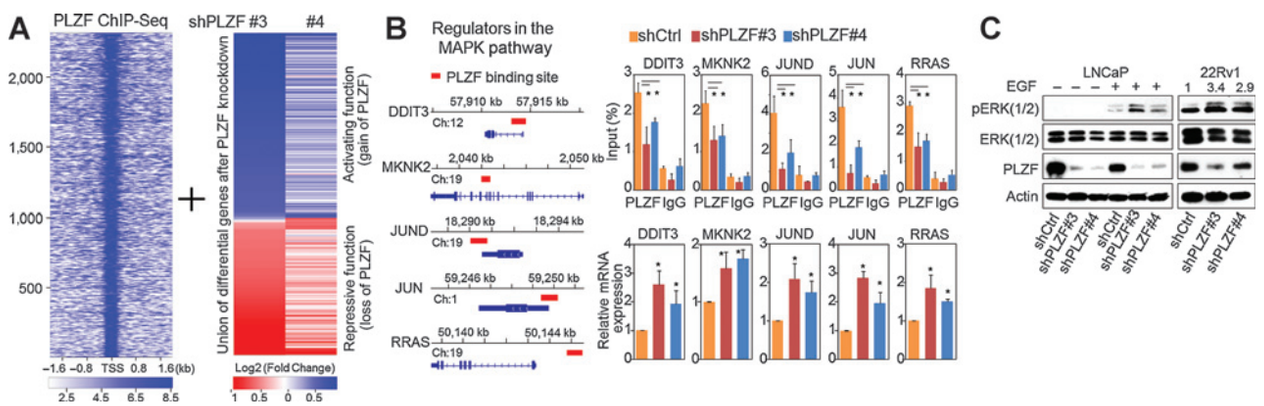

Figure 3.
Bioinformatic analysis of the PLZF transcriptional program. A, heat maps of PLZF ChIP-seq signal ±2.0 kb around the PLZF peak summit in LNCaP. The color scale indicates average signal. The numbered index of PLZF peaks is shown to the left. A cluster of differentially expressed genes in the LNCaP with stable knockdown of shPLZF# 3 or 4. B, KEGG pathway analysis of PLZF-repressed genes. PLZF direct targets are highlighted in bold red. C, Left, schematic graph shows the PLZF-binding sites (red bars) within the PLZF target gene loci as defined by PLZF. Right, ChIP-seq in LNCaP cells. ChIP and qRT-PCR validation of PLZF binding and gene expression to selected PLZF targets. Values were the mean ± SD ($n \geq 3$); *, $P < 0.05$. D, Western blot of LNCaP and 22Rv1 was used to measure PLZF expression and ERK1/2 activity with or without EGF (10 ng/mL) stimulation.

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consistent with the notion that ADT suppresses serum and tissue androgens and results in diminished AR pathway activity (9).

The subcellular localization of PLZF is mainly in the nucleus where it achieves its transcriptional repression by binding to the regulatory elements in the promoter region of the target genes (7). In order to uncover PLZF-regulated transcriptome, we defined the PLZF cistrome using PLZF ChIP-seq and gene profiling data sets (Fig. 3A; Supplementary Tables S1 and S2). Next, we investigated the potential biologic consequence of PLZF suppression. Genes whose expression was upregulated in PLZF-depleted LNCaP cells were subjected to bioinformatic pathway analysis. KEGG analysis revealed that PLZF-repressed genes were significantly enriched in the MAPK signaling pathway, including 5 genes with PLZF-binding sites, *RRAS*, *MKNK2*, *DDIT3*, *JUND*, and *JUN* (Supplementary Table S3). ChIP- and qRT-PCR confirmed that these genes are part of the PLZF-repressed cistrome (Fig. 3B). More importantly, PLZF knockdown substantially induced phospho-ERK1/2 expression upon EGF stimulation in LNCaP (Fig. 3C, left). We also observed elevated levels of phospho-ERK activity in PLZF-depleted 22Rv1 cells (Fig. 3C, right). The inhibitory effect of MAPK inhibitors (UO126 and AZD6244) on PLZF-depleted LNCaP was assessed. Cells with PLZF depletion responded better to MAPK inhibitors as compared with shCtrl, implying that the MAPK pathway may be activated due to loss of PLZF expression (Supplementary Fig. S4).

Although our results suggest that PLZF regulates ERK1/2 activity, it is unlikely that the mechanism underlying ERK activation only depends on PLZF transcriptional modulation. Moreover, PLZF has been shown to regulate a variety of downstream targets at the posttranslational level (13). PLZF-regulated intracellular signaling molecules may also cross-talk with other regulatory pathways. Nonetheless, taken together, our data suggest that suppression of PLZF may permit sustained prostate cancer cell growth under conditions of androgen deprivation in part by de-repressing key tumorigenic mechanisms, such as ERK1/2 signaling. This finding may partly explain and is in agreement with previous findings that MAPK signaling is upregulated in some CRPC murine models and patient-derived tumor samples (14–16). Thus, in the subset of patients with low PLZF expression including genetic loss, MAPK pathway inhibition may be of particular importance.

To begin to explore the potential effect of PLZF loss on AR-targeted therapy, we evaluated the impact of enzalutamide on the growth of prostate cancer cells in the absence or presence of PLZF knockdown. As expected, enzalutamide completely killed the shLuc-silenced cells when cultured in the regular conditioned medium. PLZF-depleted cells showed the ability to grow, although to a lesser extent, even in presence of enzalutamide (Fig. 4A). When we conducted the same experiment culturing the cells in androgen-deprived medium (charcoal-stripped serum, CSS), we observed a similar growth pattern. While shLuc cells remained quiescent in absence of androgens, shPLZF cells showed slight sensitivity to enzalutamide at early time point, becoming progressively resistant to the drug at late time points (Fig. 4B). To determine whether the presence of AR is required for PLZF-dependent growth, we introduced an Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible shAR in the shPLZF-stable LNCaP cells. Strikingly, our data showed that PLZF loss enables LNCaP cells to proliferate even in the absence of androgens or AR expression (Fig. 4C). These results imply that PLZF inactivation may be a key factor in the development of resistance to AR-directed therapeutics, such as enzalutamide. Collectively, our data suggest that PLZF suppression or genetic loss may underlie a novel mode of resistance to ADT, wherein an AR-repressed oncogenic program facilitates residual prostate tumor cells to adjust to castrate levels of androgens to survive or grow.

In view of the AR-dependent mechanisms for CRPC development, ADT may directly or indirectly activate an AR-repressed network, although we cannot completely exclude the involvement of oncogenic activation mediated by persistent AR expression in residual prostate tumors. Accordingly, we report that the upregulation of the PLZF-repressed oncogenic program is an acquired mechanism in response to ADT and that genetic loss of

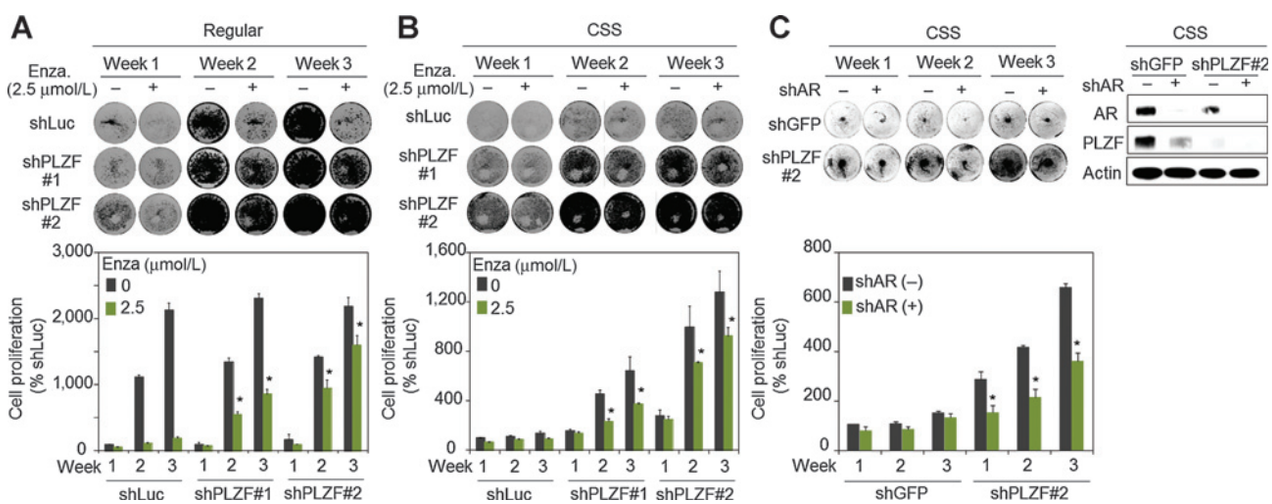

Figure 4.

PLZF depletion alters the growth-inhibitory effect of enzalutamide. LNCaP cells with or without PLZF knockdown were cultured in 5% FBS (A) or CSS medium (B) and treated with or without 2.5 μmol/L of enzalutamide or IPTG-inducible shAR knockdown (C), followed by CV staining at the time as indicated. Each column was relative to the corresponding first column and shown as mean ± SD ($n \geq 3$); *, $P < 0.05$.

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PLZF in the course of disease is important molecular event in the emergence of CRPC and development of resistance to ADT and perhaps enzalutamide. Presumably, prostate cancer genomic and transcriptomic information may permit better molecular classification and provide new insights into the mechanisms of resistance to androgen/AR signaling blockade, thus aiding the design of future therapeutic combinations to overcome drug resistance.

Disclosure of Potential Conflicts of Interest

M. Brown reports receiving a commercial research grant and is a consultant/advisory board member for Novartis. L.A Garraway reports receiving a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member for Boehringer Ingelheim, Foundation Medicine, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: C.-L. Hsieh, G. Botta, E.M. Van Allen, L.A. Garraway, P.W. Kantoff

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-L. Hsieh, G. Botta, E.M. Van Allen, L.A. Garraway, P.W. Kantoff

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